

Persistence of Adenovirus 5 in Guinea Pigs

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One intracardiac inoculation of adenovirus 5 in guinea pigs leads to virus persistence in different organs, viz., 5 days in lungs and liver, 14 days in blood and lymph nodes, and 56 days or more in the spleen. After cultivation of tissue cells for 1 week, virus was recovered from blood, lymph nodes, or spleen lymphocytes, but virus could be detected directly in cells only when organs were removed within 48 h of inoculation. To determine how the virus persisted in low concentrations and as a latent infection, spleens were primarily selected for study by three techniques: homogenization of spleens, suspended Maitland fragment cultures, and *in vitro* cultivation of spleen cells. The last procedure showed virus in fibroblast-like cells (probably macrophages or reticuloendothelial cells) for 56 days after infection of guinea pigs. With other methods, the virus was found only within the first 2 days after inoculation.

Association of adenoviruses with human lymphoid tissues was commonly found, especially with serotypes 1, 2, 5, and 6 in adenoids and tonsils of children, without evidence of clinical illness (2, 5, 6, 16-20). The virus was usually recovered after growing tissue fragments *in vitro* and persisted for a long period.

The induction of experimental latent infection has been used in attempts to explain the low concentration of virus in natural latent infections (14). In this situation, it was possible to isolate adenovirus 5 from rabbit spleen, by using the Maitland fragment culture technique, for up to 2 months after inoculation (14) or from disrupted spleen and lymph node cells inoculated into human embryo kidney cultures for up to 12 months (15).

The present investigation was designed (i) to find out whether latent adenovirus 5 infection was a common event by studying the virus after experimental inoculation of guinea pigs, and (ii) to determine whether lymphocytes carried the virus.

MATERIALS AND METHODS

Animals, virus, and cell cultures. Forty-eight male guinea pigs, 6 weeks old and weighing 250 g, were inoculated intracardially with a single injection of 8×10^6 infective doses (ID).

Adenovirus 5 (human strain 171, kindly given by R. J. Huebner) had four passages in KB cells and seven passages in HeLa cells. The titer of the inoculum was $10^{7.6}$ ID/ml (equivalent to $10^{8.6}$ plaque-forming units/ml).

HeLa cells were maintained in monolayer culture in Spinner minimal essential medium containing 10%

inactivated bovine serum. The virus was propagated in HeLa cells maintained in minimal essential medium containing 2% inactivated fetal calf serum. For virus isolation, HeLa cell cultures growing in test tubes were prepared, each tube being seeded with 30,000 cells in 1 ml of medium with 2% fetal calf serum and maintained for 14 days with medium changes every 4 days. They were regularly observed for cytopathic changes. The cultures, without sign of cytopathic effect, were freeze-thawed and then inoculated into new tubes of HeLa cells. Three passages corresponding to an incubation period of 42 days were made before the cultures were discarded as negative.

Isolation of adenovirus from various guinea pig organs. Thirty-three guinea pigs were inoculated and divided into 11 lots of three animals, which were killed after 1, 4, 12, and 24 h and then 2, 5, 7, 14, 28, 42, and 56 days from the time of infection.

Various organs (spleen, liver, and lungs) were taken aseptically and washed three times in phosphate-buffered saline.

Each spleen was divided into three fragments. One fragment was minced with scissors and then homogenized in 2 ml of nutrient medium (minimal essential medium + 2% inactivated fetal bovine serum). The suspension was treated with 0.5% sodium deoxycholate at 4 C for 16 h to solubilize the membranes and then centrifuged at $2,000 \times g$ for 15 min to remove debris (9). The supernatant was the inoculum for isolation of the virus and was kept at -20 C (0.2 ml per tube). The second fragment was cut into 20 pieces of 1 mm³ and maintained for 6 weeks in 2 ml of medium composed of bovine amniotic fluid and 5% inactivated horse serum, according to the method of Maitland and Maitland (10). The medium was replaced every week, and finally the fragments were homogenized in a Potter-Elvehjem-type homogenizer. Media and cell homogenates were stored at -20 C before inoculation of HeLa cells (0.2 ml per tube). The

third fragment was treated as described for separation of lymphocytes (next section). The cell suspension contained different types of cells. The cells which were adherent to the substrate after 12 h of incubation, identified as macrophages (7), were maintained and developed for at least 40 to 105 days. Media were kept at -20°C before inoculation of 0.2 ml into HeLa cell tubes.

For liver and lungs, two methods were used: disruption of fragments of organs with the Potter-Elvehjem-type homogenizer, and cultivation of dissociated cells from fragments minced with scissors.

Recovery of adenovirus from guinea pig lymphocytes. Purified lymphocytes were prepared from lymph nodes, spleen, and the peripheral blood of guinea pigs infected for various periods of time.

Experiments were carried out on five lots of three guinea pigs. Various lots were killed 12 and 24 h and 7, 14, and 28 days after inoculation. Spleens and lymph nodes were removed aseptically and washed three times in phosphate-buffered saline. Precrural, mediastinal, and axillary lymph nodes were used.

The fragments were minced with scissors. The cells were dissociated in 2 ml of nutrient medium composed of RPMI 1640 (Gibco) and 20% fetal bovine serum. The cell suspension containing different cell types was filtered through gauze to eliminate aggregates and tissue debris and then centrifuged at $2,000 \times g$ for 2 min. Erythrocytes were lysed by hypotonic Hanks solution. After centrifuging at low speed, the cell pellet was resuspended in nutrient medium. After 12 h of incubation at 37°C , a few cells adhered to the substrate. In the medium which was removed, most of the cells were lymphocytes. Cell viability was estimated by vital staining (crystal violet or C trypan blue). After centrifugation, cell counts were obtained in a hemocytometer. Duplicate samples containing 2×10^6 cells were prepared and incubated in nutrient medium for 1 week in suspension.

Samples of cell homogenates, media, and cell cultures were stored at -20°C before inoculation into HeLa cell tubes (0.2 ml per tube).

Blood lymphocytes were separated according to the technique described by Carraz et al. (1) by filtration of blood on nylon fibers. After addition of dextran and 30 min of sedimentation at 37°C , the top phase was removed and centrifuged for 10 min at $1,200 \times g$. The pellet was resuspended in hypotonic Hanks solution to lyse erythrocytes. Cells were rewashed and counted before culture in nutrient medium.

Identification of the virus recovered from guinea pigs. Samples with a positive cytopathic effect on HeLa cells were typed by a neutralization test using rabbit antiserum against adenovirus 5.

RESULTS

Persistence of adenovirus 5 in guinea pig spleens. When guinea pigs were inoculated intracardially with adenovirus 5 (8×10^8 ID), the virus persisted in the spleen for at least 56 days. Three different techniques were used to isolate the virus: fragment homogenization, fragment culture in suspension (Maitland

method), and in vitro cultivation of dissociated cells with a monolayer culture (Table 1).

After homogenization of a spleen fragment and inoculation into sensitive HeLa cells, a cytopathic effect was observed only in samples removed from animals which had been infected 2 days before. The virus concentration was assayed after treatment of the homogenate with sodium deoxycholate and centrifugation on a discontinuous density gradient with cesium chloride ($\rho = 1.3$ and $\rho = 1.6$). It was shown that the amount of recovered virus from spleen was very low and decreased with time after infection of guinea pigs: 10^6 ID after 1 h, 4×10^3 ID after 4 h, and 3×10^3 ID after 24 h. No virus could be detected 5 to 56 days after inoculation.

With fragment cultures in suspension, the virus was isolated from media harvested from spleen samples taken 1, 4, 12, and 24 h after inoculation. When spleens were removed later (after 2 to 56 days), no cytopathic effect was observed even after three blind passages on HeLa cells.

The technique of cultivation of dissociated cells allowed the recovery of infectious virus in each spleen for 56 days after inoculation into guinea pigs. Cultures went through three successive steps. After 12 h of incubation at 37°C of unpurified cell preparations, a few cells adhered to the glass, and some were identified as macrophages according to their morphology and preferential staining with neutral red. After 5 to 6 days of incubation at 37°C , cells were confluent and two kinds of cells were detected: macrophages and fibroblasts. Both cells were seen until day 14. When spleens were removed 1

TABLE 1. Results of the three methods used for isolation of infectious adenovirus 5 from guinea pig spleen cells^a

Time after inoculation	Cell homogenate	Maitland culture	Long-term culture
1 h	10^6 ID	++	++
4 h	4×10^3 ID	++	++
24 h	3×10^3 ID	++	++
2 days	+	—	++
5 days	—	—	+
7 days	—	—	+
14 days	—	—	+
28 days	—	—	+
42 days	—	—	+
56 days	—	—	+

^a Symbols: ++, Virus detected after one passage on HeLa cells (at least 1 ID/0.2 ml); +, virus detected after two or three passages on HeLa cells (at least 1 ID/0.2 ml); —, no virus detected after three passages on HeLa cells (less than 1 ID/0.2 ml).

h to 2 days after inoculation, the cell cultures set up showed detachment of cells between 40 and 56 days of cell culture; when spleens were removed 2 to 56 days after inoculation, cell detachment of the cultures occurred between 73 and 105 days. The medium was removed every week and replaced by fresh medium.

The infectious virus was discontinuously recovered from media, without any relation to cell shape. The amount of virus was very low and decreased with time after inoculation of the guinea pig. In early samples, the virus was demonstrated at the first passage on HeLa cells, which suggested that a rather significant amount of virus was released in tissue culture media. In late samples, three passages in HeLa cells were necessary to detect the virus, which indicated that the virus concentration was relatively lower.

Cultivation in vitro of dissociated spleen cells from 10 uninfected guinea pigs did not lead to recovery of virus capable of growing in HeLa, KB, green monkey kidney, or rhesus monkey kidney cells.

Persistence of adenovirus 5 in guinea pig lymphocytes. Adenovirus 5 was sought in guinea pig lymphocytes from spleens, lymph nodes, and blood. The persistence of the virus varied with organ origin: at least 28 days in spleens, and 7 to 14 days in blood and lymph nodes. The purity of the lymphocyte preparation was checked by staining with May-Grunwald-Giemsa.

In spleen, the virus was recovered in freshly isolated homogenized cells in samples taken within 2 days after guinea pig inoculation (Table 2). When spleens were removed later than 2 days, the virus was detected in cultures of lymphocytes at 37 C in maintenance medium during 1 week.

For blood and lymph node lymphocytes, the virus was recovered 7 to 14 days after inoculation of guinea pigs and cultivation during 1 week in maintenance medium (Table 3).

Persistence of adenovirus 5 in guinea pig livers and lungs. Infectious virus was isolated from liver and lungs of infected guinea pigs within 5 days after inoculation.

Two techniques were used, cell homogenization and culture of isolated cells, and both gave the same results. It suggested that the adenovirus 5 was circulating in guinea pigs, and it was necessary to cultivate cells to detect the virus (Table 4).

Characterization of experimental infection of guinea pigs with adenovirus 5. The properties of recovered adenovirus 5 from guinea pig organs after passages on HeLa cells were

TABLE 2. *Isolation of infectious adenovirus 5 from guinea pig spleen lymphocytes*^a

Time after inoculation	Cell homogenate	Culture in suspension
12 h	++	++
24 h	+	++
2 days	+	+
7 days	—	+
14 days	—	+
28 days	—	+

^a Symbols are as in Table 1.

TABLE 3. *Isolation of adenovirus 5 from guinea pig blood and lymph node lymphocytes*^a

Time after inoculation	Blood lymphocytes		Lymph node lymphocytes	
	Cell homogenate	Culture in suspension	Cell homogenate	Culture in suspension
24 h	—	—	—	+
7 days	ND	+	—	+
14 days	—	+	—	+

^a Symbols: +, Virus detected after two passages on HeLa cells (at least 1 ID/0.2 ml); —, no virus detected after three passages on HeLa cells (less than 1 ID/0.2 ml). ND, Not done.

TABLE 4. *Isolation of infectious adenovirus 5 from livers and lungs of infected guinea pigs*^a

Time after inoculation	Livers		Lungs	
	Cell homogenate	Mono-layer culture	Cell homogenate	Mono-layer culture
1 h	+	ND	+	ND
12 h	+	ND	+	ND
24 h	+	++	—	++
5 days	—	+	—	++
7 days	—	—	—	—
14 days	—	—	—	—

^a Symbols are as in Table 1. ND, Not done.

checked by hemagglutination, complement fixation, and neutralization tests. Recovered virus appeared to be identical with inoculated virus.

Animals acquired antibodies against adenovirus 5 as demonstrated by neutralizing, complement-fixing, and hemagglutination-inhibiting tests. The titers of antibodies were low—1:16 to 1:64.

After inoculation of the virus, the guinea pigs did not show any clinical illness and their temperatures were within the normal range.

DISCUSSION

In natural latent infection with adenovirus, the virus was recovered from cultured lympho-

cytes prepared from human tonsil and adenoid specimens (20) and also from the peripheral blood of patients with aphthous lesions (13). These findings are highly suggestive that lymphocytes may be infected with adenovirus and that they can carry the virus.

Studying the persistence of adenovirus 5 in rabbit spleen (14), it was shown that the isolation of the virus was possible for 2 months after infection with the Maitland technique and for only 3 days with the spleen homogenate method. In some cases, adenovirus 5 was detectable in disrupted cells of rabbit spleen and lymph nodes for 1 year after inoculation; but human embryo kidney cells—the most sensitive cells to human adenovirus—were used to isolate the virus (15).

In our study, experimental latent infection was produced in 48 guinea pigs with human adenovirus 5. The virus was recovered from spleen, lymph nodes, liver, lungs, and blood. The persistence of the virus was demonstrated in lymphocytes after cultivation and in long-term cultures of spleen fibroblasts (reticuloendothelial cells and macrophages). When cultures from 10 uninfected guinea pig spleens were kept under the same conditions, no contamination was detected either from adenovirus 5 or from other viruses which could multiply in human or in simian cells.

The virus recovered from early samples after inoculation of guinea pigs could be simply associated with different blood cells and then could circulate in the organism, as indicated for other viruses (11). In later samples, antibodies may prevent detection of virus, as it has been suggested in some natural human infections with adenovirus (5, 17). From previous observations on the susceptibility of monkey kidney cells to poliovirus, *in vitro* and *in vivo* (8), it is possible that some factor could be present in the organs, *in vivo*, which prevented the adenovirus from multiplying. The cultivation *in vitro* of the cells might have freed them from the influence of such a factor.

There is no doubt that the virus recovered from long-term cultures was localized in spleen cells. The persistence of the virus can be explained in two ways. First, the virus could be located within the cell so that it is protected from antibodies, from the action of interferon (4). The repeated media changes and cultivation *in vitro*, however, may well result in cell changes which activate the virus and its release. Second, the virus present in some cells was incomplete, and such cells could contain viral genome information so that in cell culture, after

modification of cell metabolism (12) and by addition of cell growth factors (3), the virus could replicate completely and produce infectious virus.

From our data, it can be stated that adenovirus 5 was persistent in infected guinea pig spleens for 2 months, that the virus was detectable only in long-term cultures, and that the amount of virus was very low. Moreover, lymphocytes were able to carry the adenovirus.

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